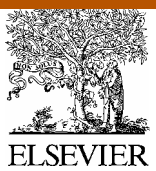

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A Protein Microsensor by Detection of Leakage Current from Interaction between an Electrolyte-Entrapping Liposome and Protein

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Abstract

We have newly made a specific biomolecule microsensor measuring leakage current and impedance with minute liposome solution that interacts with biomolecules such as protein. The microsensor was fabricated with a Si surface-bulk-micromachining process. $K_4[Fe(CN)_6]$ solution was entrapped inside of liposome of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholline (DPPC) and Carbonic Anhydrase from Bovine (CAB) was used as added protein for the interaction. A clear leakage in nA order was observed for the CAB, indicating the microsensor detects the CAB through the liposome-protein interaction. The detectivity increased by about 7 times and the volume of solution was decreased by about 10,000 from the previous result.

Keywords: Leakage current; Liposome; Electrolyte; Protein; Interaction

1. Introduction

It has been found so far that the liposome involving inside electrolyte ions such as iron ferrocyanide ($Fe(CN)_6$) can release the ions by interaction with specific biomolecules such as protein through structural change and perturbation of the shape of liposome supramolecule [1,2], leading to obtain an effective biomolecule sensor. Compared to the previous result using laboratory instruments with single and large amount of target solution [2], it is needed to have a combinatorial microarrayed approach for effective investigation of complex phenomena in the liposome-protein interaction using important biochemical parameters.

In this work, we fabricated a leakage current microsensor using Si-surface-bulk micromachining process. A micro well for a droplet of the liposome immobilized on hydrophilic thermal SiO_2 surface, and Pt sputtered film electrode were formed. This leads to make the target solution volume less than μL with good immobilization of the droplet and makes possible to measure minute solution. Leakage current was measured on the basis of coulometry.

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The released ions of finite number contribute on the current generation, which relates directly to the interaction of lipid membrane of liposome and external protein. The leakage current disappears after the ions are consumed and the interaction is finished. On the other hand, the interaction will give some change on the surface properties and structure of the liposome. Therefore, dielectric properties of the liposome were also evaluated with an impedance method, especially Cole-Cole plot was obtained.

2. Liposome and Protein

Liposome is microscopic, fluid-filled pouch whose walls are made of layers of phospholipids identical to the phospholipids that make up cell membranes. It was found that phospholipids combined with water immediately formed a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble. Water-soluble medications added to the water were trapped inside the aggregation of the hydrophobic ends; fat-soluble medications were incorporated into the phospholipid layer. In some cases liposomes attach to cellular membranes and appear to fuse with them, releasing their contents into the cell. Sometimes they are taken up by the cell, and their phospholipids are incorporated into the cell membrane while the drug trapped inside is released.
This time, $K_4[Fe(CN)_6]$ solution was entrapped inside of liposome of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholline (DPPC).Carbonic Anhydrase from Bovine (CAB) was used as added protein for the interaction. The CAB is selected as a target protein because it was conventionally used and evaluated in the experiments with normal laboratory instruments [2], so the experimental results are compared between this work and the conventional.

We can consider from the comparison whether the new approach of the microsensor is effective for improving the performance of several figure-of-merits, for example, target sensitivity, target volume and so on, as a protein sensor.

3. Measurement Principle of Leakage Current Sensor

The lipid membrane of the DPPC liposome is perturbed by the interaction with the external CAB protein, thereafter iron ion Fe^{2+} is released from $K_4[Fe(CN)_6]$ solution inside of the DPPC liposome through the perturbed lipid membrane. The Fe^{2+} ion is oxidized to Fe^{3+} and generated electron goes to the positive electrode. The series phenomena are illustrated in Fig. 1.
The leaked Fe^{2+} ion shows an electrochemical reaction in neighbor of the electrode in Eq. (1).

$$Fe^{2+} \rightarrow Fe^{3+} + e^- \quad (1)$$

The generated electron gives the leakage current in the microsensor. The leakage current is dependent on the quantity of Fe^{2+} ions leaked from $K_4[Fe(CN)_6]$ solution inside of the DPPC liposome, so this means that one can obtain a quantitative intensity of interaction between the DPPC liposome and CAB protein, such as an external CAB protein concentration, activity of the interaction.

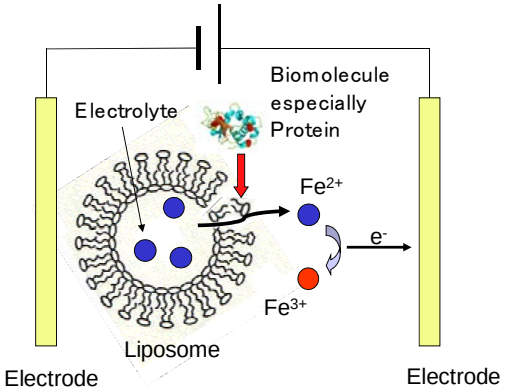

Fig. 1. Leakage current due to release of electrolyte ion from inside of liposome.

4. Fabrication of Leakage Current Microsensor

A Si wafer (280 μm thickness) with a grown SiO_2 layer (1 μm thickness) is used as an initial substrate. Major fabrication processes are illustrated in Fig. 2. At first, a micro-well is formed by TMAH anisotropic wet etching that holds a droplet of electrolyte-entrapping DPPC liposome steadily. Second, the whole surface of the well are thermally oxidized to eliminate external leakage current between the sensor electrodes, and to have a hydrophilic surface for keeping intact molecular structure of the liposome. Thirdly, Pt/Ti film (about 1 μm thickness) electrodes were formed by rf-sputtering and lift-off method. Figures 3 show photomicrographs of fabricated sensor chip. The chip size, the depth of the well and the widths of electrode are about 14 mm, 100 μm and 500 to 2000 μm, respectively.

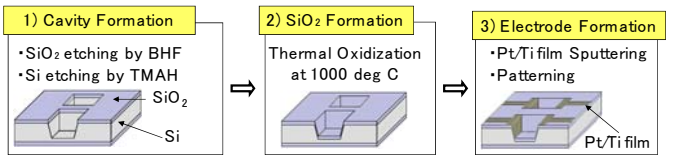

Fig. 2. A fabrication flow of leakage current microsensor.

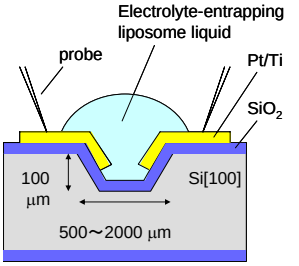

Fig. 4. A cross-sectional view of leakage current microsensor.

5. Results on Leakage Current Experiments

Figure 4 shows a cross-sectional view of the leakage current sensor where the liposome droplet is fixed in the well. Thereafter, the CAB droplet of 1 μL is supplied on the liposome manually from a micropipette. Time course of the current with 0.1 V bias between the electrodes is monitored before and after the CAB dropping with a semiconductor parameter analyzer (Agilent 4156B). In Fig. 5 is plotted the current versus time and the increased peak area (current x time) corresponds to the generated charge originated from the released Fe^{2+} ions. It is observed that certain response time exists to show the current just after dropping the CAB solution.

On the other hand, denatured CAB protein shows enhanced interaction with liposome and tends to release more the inclusion [3], and the amplification of the current is expected and obtained in Fig. 5. Then the leakage current is evaluated with DPPC liposome and denatured CAB by GuHCl as protein denaturant. Figure 6 shows leakage current dependent on the denatured CAB concentration. The concentrations of electrolyte-entrapping DPPC liposome and the GuHCl added to CAB are 100 μM and 0.5 M, respectively. Also the gap between the sensor electrode and droplet volume are 500 μm and 1 μL, respectively. The leakage current was measured as a parameter of the denatured CAB concentration (0, 0.4, 1.0 μM). It is found from Fig. 6 that the leakage current increases monotonously with the concentration, indicating the interaction increases with the denatured CAB concentration.

We also measured the droplet volume dependence of leakage current, where the concentration of the electrolyte-entrapping liposome, CAB and GuHCl were 100 μM, 0.4 μM and 0.5 M, respectively. The gap between the sensor electrodes was 800 μm, as a large volume of droplet was used. In Fig. 7 is shown the leakage current versus droplet volume. It is found from the figure that the current increases with the volume, but both are not linearly correlated

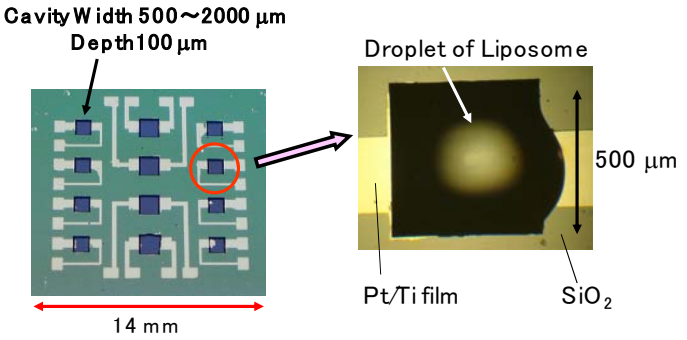

Fig. 3: Surface photographs of fabricated microsensor.

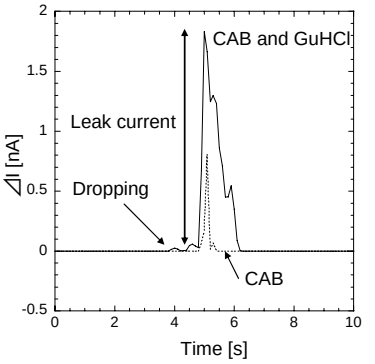

Fig. 5. Leakage current vs. time.

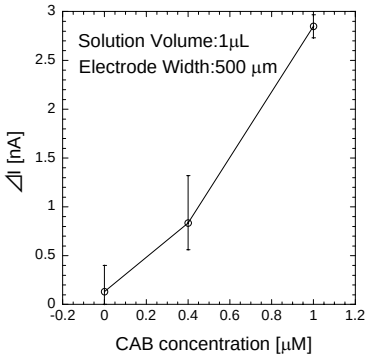

Fig. 6: Leakage current vs. added CAB concentration.

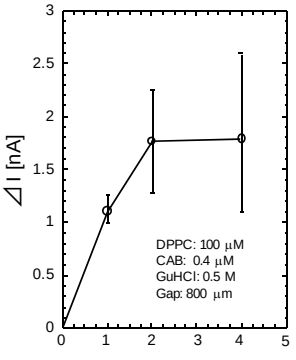

Fig. 7: Leakage current vs. droplet volume.

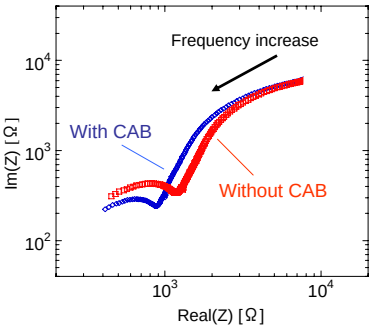

Fig. 8: Cole-cole plot.

with each other. It indicates that the phenomena of transport of generated electron due to the oxidization reaction occur in neighbor of the electrode, so the Fe^{2+} ions not near the electrode did not contribute on the generation of leakage current because the supporting electrolyte was added.Compared to the previous result [1], it was observed that the detectivity (assumed as leakage current/(CAB concentration x CAB volume)) increased by about 7 times and the volume of solution decreased by about 10,000.

6. Evaluation of Impedance and Results

From the results shown above, the leakage current generation is a phenomenon shorter than at most 1 s. It is considered, on the other hand, that after the interaction between the liposome and protein it will result in some change in surface properties and molecular structure of the lipid membrane on the liposome. And the changes are reflected to dielectric properties of the liposome. We therefore proceed to evaluate impedance characteristics of the liposome immobilized on the microsensor, as the impedance measurement is very sensitive for the dielectric properties. One of the most effective method of the characterization is Cole-Cole plot. Figure 8 shows Cole-Cole plots measured from 100 Hz to 5 MHz for with and without CAB on the microsensor. It is observed that basically there exist two kinds of RC parallel circuits (half-circled curve), which is considered to correspond conventionally electrode/solution interface reaction and liquid bulk reaction. It is also found that the curve with CAB becomes higher/lower than that without CAB for frequency lower/higher than 1 MHz. This indicates that increase in diffusion impedance due to generation of DPPC-CAB complex occurs especially for the lower frequency, which results in fusion and aggregation of the lipid membrane.

7. Conclusions

The leakage current by the interaction was successfully measured dependent on the droplet protein concentration. Also a protein denaturant of GuHCl is effective for enhancing the leakage current by promoting CAB denatured. It is indicated that the leakage current sensor makes it possible to evaluate quantitative interaction between the liposome and protein. Cole-Cole plots from the impedance analysis also showed a quantitative difference between with and without the interaction, depending on the solution impedance that results from condition and structure of liposome and its lipid membrane.

Acknowledgements

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